A CHEMOSYSTEMATIC STUDY OF *MUSA*

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By

James William Bonner

Thesis Committee:

Robert M. Warner, Chairman
James L. Brewbaker
Henry Y. Nakasone

We certify that we have read this thesis and that in our opinion it is satisfactory in scope and quality as a thesis for the degree of Master of Science in Horticulture.

THESIS COMMITTEE

Robert M Warner
Chairman

James L. Brewbaker

Henry Washington

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INTRODUCTION

The use of gel electrophoresis to separate multiple molecular forms of enzymes, termed "isozymes" by Markert and Møller (1959) produce a wide variety of band patterns in higher plants (Shannon 1968). These band patterns, known as zymograms (Hunter and Markert 1957) are thought to be products of gene segregation in a Mendelian form.

There has been extensive use of zymograms to study enzymatic activity during the development of plants. These various ontogenetic stages exhibit different zymograms, which investigators believe are due to genetic derepression or activation of synthesis during development (Scandalios 1969). These may be associated with physiological changes during maturation (Racusen and Foote 1966; Siegel and Galston 1967; Makinen 1968).

Only within the past eight years have advancements in electrophoretic and histochemical techniques made possible detailed studies on specific genera of plants. Isozymic resolution is presently so refined that the absence of one amono acid in an enzyme can be detected (Ingram 1957).

In Musa, systematic investigators have been limited somewhat, due to the wide variation in diagnostic characters used in taxonomic studies and the wide range of growing conditions in which these plants are found. There is much added confusion because of the synonymous names given to bananas in various countries (Simmonds 1970).

The objectives of this study were to determine the isozymic patterns of the peroxidases of the available clones of Musa and to possibly identify species specific patterns. Tissue specimens had to be easily available, utilizing locally available materials. The peroxidases were chosen because they have proven useful in the past in other systematic studies.

LITERATURE REVIEW

Systematics of Musa

The banana cultivars of the world are so inadequately studied that it is impossible to make a guess at the number of distinct clonal varieties in existence (Shepherd 1970). The majority of ancient cultivars are thought to have originated from cultivars of the wild M. acuminata type (N=11) (Simmonds 1966). Today the majority of banana cultivars are thought to be hybridizations of M. acuminata x M. balbisiana (Simmonds 1962; Simmonds 1966; Shepherd 1970). In New Guinea, some specific clones are thought to be hybrids of M. acuminata x M. schizocarpa (Shepherd 1970).

The first attempt to classify edible bananas was made in 1955 by Simmonds and Shepherd (Simmonds 1962). First, general sections were established, (Appendix 1). The bananas of the Eumusa section were further classified by a point system. This system gave point values, one to five, for specific characteristics of the plant; lower points tending toward M. acuminata and higher toward M. balbisiana type plants, (Appendix 2). When all observations were made, the scores were totaled. Point values 15-23 represented M. acuminata type cultivars and 26 points or more represented hybrids of M. acuminata x M. balbisiana. This system is still utilized today but the accuracy of this method may be validly questioned.

Isozymes

Gel electrophoresis, first described by Smithies (1955), provides a simple method for the detection of minute protein differences from a relatively small quantity of crude extract.

Starch gel was the medium used for his protein separations, obtaining superior resolution to previous methods due to the "molecular sieving effect" that the starch afforded. With this technique, proteins are separated on the basis of their net charge; a single amino group substitution will change the mobility of the protein and can be detected (Ingram 1957).

Hunter and Markert (1957) introduced a method for staining specific proteins. These histochemical methods have been expanded and presently a large number of specific proteins may be isolated. Along with their introduction of histochemical detection of proteins, Hunter and Markert (1957) proposed the term "zymogram" to describe the visible patterns produced after staining for specific proteins. These zymograms are very specific and are sometimes described as the "fingerprints" of a particular material.

Markert and Møller (1959) proposed the term "isozyme" to describe the multiple molecular forms of enzymes that appear after staining the starch gels. They theorized that enzymes exist in different molecular forms which display high enzyme specificity. Peroxidases were thought to have overlapping substrate specificity. This specificity was thought to be the result of each enzyme being attributed to a single gene, with the differences attributed to the genes possessed or, alternate forms of a gene that may produce a somewhat different constellation of isozymes, each species having a uniquely characteristic gene for synthesis of a particular enzyme.

Stable media with high resolving power for use in electrophoresis has long been sought. With the advent of polmerization

methodology, polyacrylamide (Cyanogum) gel has recently been introduced (Raymond and Wientraub 1959). The gel can be used in either vertical or horizontal gel apparatus and provides high resolving power as well as a molecular sieving effect. Since the introduction of polyacrylamide gel, there have been many individual systems and techniques employed. Two useful reviews are available (Brewer 1970; Chambach and Rodbard 1971).

Electrophoresis in Genetic Studies

Some earlier works involving isozyme studies in inherited electrophoretic variations were conducted by: Allen (1960), using esterases in Tetrahymena; Schwartz (1960), esterases in Maize; Tashian (1961), esterases in human erythrocytes and Bayer (1961), on human alkaline phosphatase.

The initial demonstrations of genetic polymorphism by electrophoretically different enzymes and the high frequency of detection of this polymorphism were not appreciated at first. Shaw (1965) seems to be the first to take notice of this high frequency and the usefullness of electrophoresis as a tool for mass screening of enzyme mutants. He also suggested using isozymes as genetic markers for studying structure, multiple substrate specificity and biochemical analysis of the enzyme.

An investigator could make a rough estimate of the proportion of genetic loci in the population which showed variation by determining the proportion of randomly selected enzymes which displayed electrophoretic variation (Lewartin and Hubby 1966). Before this method was developed, the genetic systems could not allow for a

realistic estimate due to the biased way gene products come to the attention of the investigator.

Electrophoresis in Systematic Investigations

Various ontogenetic stages in plant material show different isozymes. This has led investigators to believe that the differences were due to a genetic derepression or activation of genes during development (Ockerse, Siegel and Galston 1966; Siegel and Galston 1967; Scandalios, Liu and Longo 1968; Gupta and Stebbins 1969). These changes in enzyme structure may be associated with physiological changes which a tissue undergoes during maturation (Bhatia and Nilson 1969; Roberts 1969; McCrown, Beck and Hall 1969).

The use of zymograms has proved itself in systematic studies. Marshall and Allard (1969) found two codominant alleles at each locus studied in Avena barbata. Evidence indicated that the same bands were at the same linkage group. Citrus spp. studies (Goren and Goldschmidt 1966; Warner and Upadhya 1968) showed that enzyme differences were interdependent among species. Investigators indicated that each Datura species (10 studied) was isozymatically distinct for peroxidases and that no single band was common to all species (Conklin and Smith 1971). McCrown, Beck and Hall (1969) demonstrated that in the three species of Dianthus studied, the winter hardy clones developed two to four new peroxidase bands several weeks before reaching the period of hardening. Species of Fabaceae were investigated and although no definite conclusions from a systematic standpoint were reached, the usefullness of isozymes in a systematic study was noted (Thurman, Boulter, Derbyshire and

Turner 1967). Peroxidase activity in the seed coat of Glycine max was found to be highly heritable. Among similar species the peroxidase activity was qualitatively the same but quantitatively different (Buttery and Buzzell 1968). Moustafa (1963) detected different peroxidase mobilities between various leguminous plants. Crude extracts of the root nodules revealed no species specific patterns. Maize isozymes have been studied extensively and determinations of parent and hybrid zymograms have demonstrated that parental isozymes can be located in the hybrid zymogram. (Schwartz 1960; McCune 1961; Hamill and Brewbaker 1969). Nicotiana spp. investigations have revealed characteristic species patterns, with high differences in patterns between species (Hart and Bhatia 1967; Sheen 1969; Smith et al. 1970). The autopolyploids and amphidiploids of tobacco appear to be identical to the diploid parents, which suggests that peroxidase isozymes are independent of ploidy level. Also noted was that some species from different subgenera, but from the same area, displayed similar peroxidase bands. This may suggest that plants closely related in phylogeny or by geographic isolation, change peroxidase genes by mutation and selection (Sheen 1970). West and Garber (1967a, 1967b) using crude cotyledon extracts from germinating Phaseolus seeds, determined that the hybrids exhibited the parental isozymes. Heinz and Mee (1971) used clones H-37-1933 and H-50-7209 of Saccharum to show that morphological and enzyme patterns were not correlated but the peroxidase patterns did illustrate a tendency toward species specific patterns. Fifteen esterases were isolated from Solanum spp. and

there was evidence that this enzyme was controlled by three alleles E^a, E^b abd E^c) and could be traced to certain parental lines. The enzyme was independent of taxonomic status or ploidy levels (Desborough and Peloquin 1967). Self pollinating Triticum spp. displayed a high level of multiple molecular isozymes, which may be vital from an evolutionary standpoint. The broadly adaptable species displayed greater protein diversity than the narrow ranged species (Sing and Brewer 1969).

Peroxidase in Plants

Peroxidase activity and function is a highly studied field with much literature written on the subject. Saunders, Holmes-Siedle and Stark (1964), indicated that peroxidases appear to act on a large number of substrates and are involved in a large number of growth functions of the plant, which is the reason for difficulty in investigating the function of peroxidases in a plant. The initial reaction may be specific but the primary oxidation product itself may be able to react with other substances present in the system, which could not react with the peroxidase enzyme. There is clearly no one plant peroxidase, but many different peroxidases carrying out specific reactions by a similar scheme.

The peroxidase enzymes belong to the class of enzymes known as oxidoreductases, which are capable of catalyzing oxidation-reduction reactions. Peroxidase has the systematic name of Donor: H_2O_2 oxidoreductase and an Enzyme Commission Number of 1.11.1.7 (Wilkinson 1965). Peroxidase catalyzes the oxidation of compounds such as IAA (auxin), aromatic amines, leuco-dyes,

ascorbic acid and phenols such as eugenol, ferulic and sinapic acids which are substrates for lignin formation. Organic ions using nydrogen peroxide as the oxidant may also be catalyzed by the peroxidases.

Peroxidase activity has been found in most tissues studied, displaying much variation among plant tissues. The root has been described as the organ with the greatest peroxidase activity by many authors. Recently the roots of Citrus spp. were studied and again it was demonstrated that the root possessed the greatest peroxidase activity (Goren and Goldschmidt 1966). The specific areas of activity were discovered to be the root hair cell initiates (Avers and Grimm 1959).

In plant cells, peroxidases are believed to be located in the microbodies. Plesnicar, Bonner and Storey (1967) found that in mascerated mung bean, peroxidases were 90% soluble with the remainder bound to the membrane of the microbodies.

Van Overbeek (1935) demonstrated that corn tissue destroyed auxins by an enzymatic process and that this destruction was parallel to the peroxidase activity. Since that time, indoleacetic acid (IAA) oxidase has been intensely studied. Galston, Bonner and Baker (1953) proved that in peas, the enzyme involved in IAA oxidation was a peroxidase. In fact, crystalline horseradish peroxidase was also capable of exidizing IAA.

The role of peroxidase in lignification is very interesting because lignin is found in plant cells only. Several enzymes have been implicated in the synthesis of lignin. Siegel (1953, 1954, 1955)

determined that lignin could be formed when excised tissue sections from red kidney bean seedlings were incubated with eugenol and thymol in the presence of hydrogen peroxide. The reaction was effected by a thermolabile cyanide-sensitive catalyst. This inferred that a peroxidase enzyme was taking part in this reaction. There was also a positive correlation between distribution of peroxidase and the pattern of lignification. Similar results were reported using vascular tissues from the root of Vicia faba (Jensen 1955). The vascular tissue was provided with eugenol and hydrogen peroxide. De Jong (1967) provided direct evidence against the role of peroxidase in lignification within onion root xylem tissue. However, there is little doubt that peroxidases do provide an important function in the process of lignification in certain plants.

In all tissues studied, peroxidase activity has increased when the tissue is exposed to disease or mechanical injury. Following host plant invasion, protein metabolism has been shown to be altered, with an accompanied increase in the oxidative systems (Rudolph and Stahmann 1964; Tomiyama and Stahmann 1964). Increased peroxidase activity in infected plants has been noted in many species: black rot resistance in sweet potato was correlated with the production of new protein after initial infection. These new proteins were then identified as peroxidases (Vritani and Stahmann 1961; Clare, Weber and Stahmann 1966). Similar results were noted when sweet potato tissue was inoculated with vein clearing virus (Loebenstein and Linsey 1961). Phaseolus varieties have shown new and increased peroxidase activity at the site of inoculation

after being infused with virus (Staples and Stahmann 1964; Farkas and Stahmann 1966). These new peroxidases were experimentally determined to be products of ths host plant and not the virus. The non-virus experiment used lesions caused by mercuric chloride to replace the virus infusion. Peroxidase changes similar to the virus induced changes were noted (Solymosy, Szirmai and Beczner 1967). In Pinto beans inoculated with virus, the young tissue displayed peroxidase isozymes associated with old leaves, suggesting that the peroxidases were a by-product of induced sesescence (Bates and Chant 1970). Rust infected flax plants produced new and more intense peroxidase activity (Andreev and Shaw 1965). A high positive correlation was found between peroxidase activity in different organs and resistance to Phytophthora in potato plants (Fehrmann and Dimond 1967). Nicotiana species infected with P. tobaci produced increased peroxidase activity to the same pathogen (Lovrekovich, Lovrekovich and Stahmann 1968; Veech 1969). Similar responses were noted in oats (Novacky and Wheeler 1970) and clover (Stavely and Hansan 1967).

A good review of the isozyme changes displayed in various plants as a result of host-pathogen interactions is presented by Latner (1967).

MATERIALS AND METHODS

Materials

The plant materials used were field grown plants of Musa spp. obtained from the University of Hawaii's Plant Science Instructional Arboretum at the Waimanalo Research Station, Waimanalo, Hawaii. Over 50 species are listed (Warner 1970).

Approximately 10 square centimeters of mature leaf tissue was collected from the basal portion of the third leaf from the top of the plant. Collection was performed when the tip of a new, folded leaf protruded from the center of the pseudostem. No tissue was collected from fruiting plants or young suckers. These tissues were either used directly or were frozen at -2° to -4° C. No difference in peroxidase patterns were produced by freezing.

Horizontal polyacrylamide gel electrophoresis was used for the separation of the peroxidase isoenzymes. A modified procedure of Brewbaker et al. (1968) was developed and used.

Equipment: The power supply consisted of a Heathkit IP-32 which could provide a direct current up to 400V at 100mA. The unit can power two gels connected in a parallel sequence, operating at about 250V. at 70 - 90 mA.

The gel trays (18 cm x 20 xm) were made from 3.2 mm clear plexiglass, with a rim 3.2 mm x 3.2 mm. The tray accommodates about 100 ml of gel, 3.2 mm thick.

The following bulk solutions of buffer were prepared:

Buffer A (pH 8.1) 0.025 M lithium hydroxide (16 gm/ 15.2 l. distilled water)
0.200 M boric acid (188 gm/ 15.2 l. distilled water)

Buffer B (pH 8.3) 0.010 M citric acid (29 gm/ 15.2 l. distilled water)
0.065 M Trizma base (120 gm/ 15.2 l. distilled water)

The buffer tanks were constructed from plastic food containers (22 cm x 7 xm x 7.5 cm), with a slot approximately 3 cm wide cut in the top extending the length of the tank. Two types of electrodes were used. One consisted of 12 inches of platinum wire (26 gauge) wrapped loosely around a glass rod 20 cm in length. The other type consisted of a mat of stainless steel screening (1/16 inch mesh) placed along the bottom and extending 4 cm up the sides of the tank. Buffer solution A was used in the tanks as the electrolyte (pH 8.1). A thin sponge 18 cm square was saturated in the buffer solution. One edge was placed in the electrolyte and the other was laid about 4 cm over the edge of the gel to act as the conductor for the current. The buffer was replaced after one or two runs.

Refrigeration is customarily provided to this system to dissipate the heat that is generated when the system is operating. This was accomplished by placing the buffer tanks and gel tray in a refrigerator and connecting the power supply by wire running through the insulating gasket to the buffer tanks.

Methods

Procedure: A 7% polyacrylamide gel was prepared in the following manner: 7 gm of Cyanogum (electrophoresis grade) was dissolved in 90 ml of Buffer B plus 10 ml of Buffer A (final pH 8.2).

To catalyze the formation of the gel, 0.30 sl of N, N, N', N'-tetramethylethylenediamine (TEMED) and 2 ml of 10% ammonium persulfate were rapidly added to the Cyanogum-buffer solution. The solution was then immediately poured into a prepared gel tray. The solution hardened in about 15 minutes. Prior to pouring the gel solution, an aluminum plate, consisting of a series of inverted "V" cuts along a straight edge was placed along the origin. When the gel hardened, the plate was removed leaving a series of 0.7 cm slits in the gel.

Samples two and four mm in diameter were taken from the collected leaf tissue. The tissue was treated with two drops of saline solution (0.8% sodium chloride plus 0.2% sodium nitrate solutions) and crudely macerated with a pestle on a sheet of aluminum foil. Treatment of the tissue with ultra-centrifugation plus saline, saline solution plus polycar AT and saline plus 0.25 M ascorbis acid were tried but later abandoned, since they provided no improvement in resolution of peroxidases. Proteins diffused readily into the saline solution and were drawn off into filter paper wicks (0.1 cm x 0.5 xm). The wicks were placed directly on the macerated tissue or the extract was taken into the wick through a piece of lens paper. The latter technique resulted in better resolution.

The wicks were then placed into the slits in the gel. With the wicks in place, the surface of the gel was blotted with a paper towel to remove surface moisture. Since the front was almost invisible in this type of gel, markers were used to help locate it. Wicks

soaked in 1% Ponceau solution were used for this purpose. A small piece of Saran wrap was placed over the surface of the gel leaving 4 cm of each end exposed. This prevented gel desiccation during the run. The gel was then placed on top of the buffer tanks which were filled with Buffer A to a depth of 2 cm. The sponge bridges, saturated with Buffer A, were laid over the exposed ends of the gel. A glass plate (18 cm x 18 cm) was added to hold the sponge bridges in good contact with the surface of the gel.

When the current was added to this system, confirmation of current flow was made by observation of gas bubbles evolving from the electrode. The gel was removed when the borate front reached about 4 cm from the anodal end of the gel.

The gels were allowed to run about 4 hours at 200 to 250 V. at a temperature of 7°C. During this period the marked borate front traveled about 8 cm toward the anodal end of the gel. At the end of the run, the glass plate, sponge bridges and wicks were removed from the gel. The gel was then removed from the tray and placed in a staining solution.

A staining solution was prepared by mixing 80 ml of 35% ethanol, 9 ml of glacial acetic acid and 1 gm of benzidine dihydrochloride and heating slightly to completely dissolve the benzidine dihydrochloride. The solution was then cooled and 3 ml of 3% hydrogen peroxide was added immediately prior to the immersion of the gel in the staining bath. The intensity of the stain could be manipulated by the length of time the gel was

immersed in the solution. Normally one-half to one and one-half minutes were sufficient.

The zymograms were then interpreted by placing the gel on a translucent plexiglass surface which covered the light source of two 40 watt incandescent light bulbs. The bands were read directly from the gels.

RESULTS AND DISCUSSION

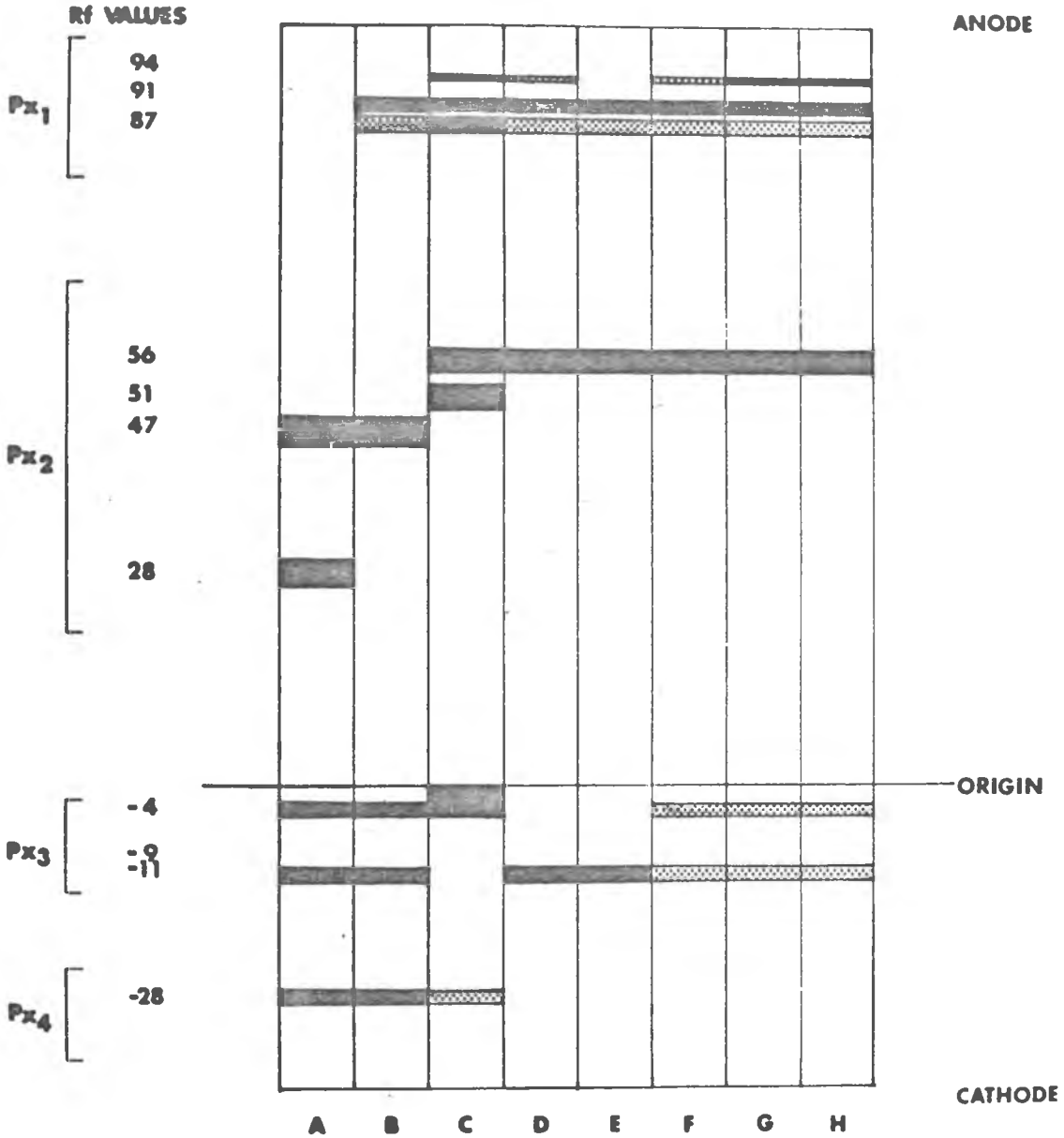
Tissue Polymorphism

A series of different plant organs were used to determine the best material for representing peroxidase activity in bananas (Figure 1). Peroxidase bands could be resolved on the cathodal side of gels by using root tissues, but there was great difficulty in obtaining these tissues. There seemed to be little difference in peroxidase activity between petiole, leaf rib, leaf margin or leaf tip. Therefore, the leaf tissue was selected as the most practical material to be utilized in a chemosystematic study. Pseudostem tissue was tested but the abundance of sap and the phenoloxidative darkening on exposure to light and air, interfered with the resolution of the bands. Root primordia exhibited good resolving properties but were awkward to collect. Another problem with root primordia was the difficulty in obtaining uniform sample size.

pH Variation

A series of samples were collected from parts of various banana plants. Approximately 100 gms (fresh weight) of a particular organ or tissue was weighed, placed in a Waring blender to which 100 ml of distilled water was added. The contents were blended for 30 seconds and filtered for use. The pH values of the blended tissue samples ranged from 7.05 to 7.20. An electrophoretic run was set up with the buffer tanks at pH 8.1 and the acrylamide gel at pH 7.1. The results were not satisfactory because anodal mobility was slowed to the point where the borate

Figure 1. Zymograms of Different Areas of the Same Plant ('Haikea'). (A) Young Root Tip. (B) Old Root (Mid Section). (C) Corm Tissue. (D) Leaf Petiole. (E) Leaf Mid0Rib. (F) Leaf Apex. (G) Center of Leaf. (H) Leaf Margin


Rf VALUES
ANODE
Px1
94
91
87
Px2
56
51
47
28
ORIGIN
Px3
-4
-9
-11
Px4
-28
CATHODE
A
B
C
D
E
F
G
H

front, marked by the Ponceau solution, was almost twice the distance from the origin as were the peroxidase bands.

In the following experiment, acrylamide gels were adjusted to a series of pH values ranging from 9.0 to 6.5. The electrolytic solution in the buffer tanks remained the same (pH 8.1). At the higher pH's, the mobility of the anodal peroxidases increased to the optimum of about 8.0 to 8.5. The lower pH values decreased the mobility of the anodal peroxidases. The cathodal peroxidase bands became visible only below pH 8.0 and increased in mobility as the pH became lower. It was also noted that a longer (2 to 3 minutes) staining period was necessary to achieve the same results at a low gel pH as compared with the rapid (30 to 60 seconds) staining period of the higher pH's. The best pH for detecting anodal and cathodal peroxidase, using a single gel, was in the range of 7.5 to 8.0. A gel of pH 8.2 was selected and proved extremely satisfactory.

While establishing an optimum pH to run the acrylamide gel, it was discovered that sample size was important in obtaining high resolution of the peroxidase bands. Consistant sample sizes from the leaves were obtained by using standard size cork borers. After running a series of sizes, best results in the Px_2 region were obtained with a number 2 borer (diameter 2 mm). The number 5 borer offered the sample size best for resolution in the Px_1 and Px_4 regions. At the onset of the investigations, standard practice was to run duplicate gels, one with large (#5) borer size samples and the other with the small (#2) size samples.

Basic Musa Leaf Peroxidases

Four distinct regions of peroxidase staining were discovered. They proved to be easily identifiable and fairly consistent throughout the genus. They were given the symbols Px_1 (peroxidase zone 1), Px_2, Px_3, Px_4 and will be considered here in detail.

R.f. (relative to front) values were assigned to all bands (Table 1). The value was calculated by dividing the distance from the origin to the particular band by the distance from the origin to the borate front and converted to percentages.

Px_1: Px_1 was the most rapidly migrating Musa peroxidase, placing it among the most rapidly moving reported or observed in plants (Brewbaker, personal communication). This indicated the probability of a very small molecule. Four bands were discovered in the Px_1 region, always in the same relative position to each other. This zone was the lightest staining of the four regions and the large samples were desirable for good resolution. Relative front positions of the Px_1 bands varied depending on the duration of electrophoresis, with approximate R. f. values for the four bands of 81, 87, 91 and 94 (Table 1).

Px_2: The Px_2 region was characterized by several heavy banks. Of these a standard band ("M", or medium) was located at R. f. 51 on the gels. A slower band "S" (R.f. 47) was observed in a few strains while the unusually slow band, VS (R.f. 43) was observed only in M. coccinea. Similarly, a fast band, F (R.f. 56) and a very fast band (VF (R.f. 59), characterized only a small number of the clones. In specific hybrid clones, two bands were

Table 1. Average R.f. Values for Musa Leaf Peroxidases in Relation to the Borate Front

Peroxidase Zone	Designation	Approximate R.f. Values	Comments
Px_1	F	94	Fastest band observed.
	M	91	Major band in this zone
	S	87	Light staining.
	VS	81	Rare.
Px_2	VF	59	Only in ABB genotypes.
	F	56	Present with B genome.
	M	51	Major reference band.
	S	47	Rhodochlamys and rare cases.
	VS	43	M. coccinea.
	VVS	20	Only in one clone 'Lacatan'.
Px_3	S	-4	Heavily stained.
	M	-9	Poor resolution.
	F	-11	Easily diffused.
Px_4	M	-28	Either present or absent.

present in this zone. In two 'Lacatan' plants an extremely slow, VVS (R.f. 20), band was observed. Generally the Px_2 zone was the most intensely stained area, staining almost immediately on immersion. Gel or time variations caused minor differences in the position of the bands. For these reasons, this was the major region studied when comparing clones.

Px_3: This region was the slowest moving of all regions studied. Staining was moderate with three bands observed. It was noted that the medium band (R.f. -9) and the fast band (R.f. -11) were never found together but were often associated with the slow (R.f. -4) band. The closeness of these bands to the origin suggested large molecules.

Px_4: Px_4 was the fastest moving region on the cathodal side of the gels. There was only one band observed (R.f. -28) exhibiting a light and slightly diffused staining pattern. This region only displayed activity in some diploid clones. An unusual occurence was noted in one plant of 'Walha'.

Band Values: The R.f. values from a series of gels were used to calculate a simple statistical analysis. This was done to provide a mathematical grouping of the peroxidase bands. Band differences were generally obvious by inspection, or by comparison with selected control or standard reference bands on the gel. For the purpose of reporting these bands, empirical limits were sought.

The R.f. values for a sample of 100 bands identifiable as medium Px_2 were measured and subjected to variance analysis:

$n = 100$
$\bar{x} = 51.323$
$Ex = 513.3$
$C.f. = (Ex)^2/n = 263,405.0$
$s^2 = Ex^2 - C.f. = 264,484.5 - 263,405.0 = 1,079.5$
$s = \sqrt{Ex^2/n-1} = \sqrt{1,079.5/99} = 3.18$
Coefficient of Variation = $C = s/\bar{x} \times 100 = 3.18/51.32 \times 100$
$= 6.19\%$

The low value for the coefficient of variation indicates there is not a great deal of variance in the position of the medium Px_2 bands within the population.

The R.f. values for the other five bands were averaged, using 100 samples per band (Table 1). The bands were abbreviated as follows:

Very Fast	=	VF
Fast	=	F
Medium	=	M
Slow	=	S
Very Slow	=	VS
Very, Very Slow	=	VVS

These abbreviations were then used to establish a chart (Table 2) to compare the peroxidase bands of the available Musa clones.

Table 2. Present Systematic Classification of Musa and Observed Peroxidase Bands.

Section Species 1/ Clone	Px_1	Px_2	Px_3	Px_4	Zymogram 2/ Number
Callimusa					
M. coccinea	--	VS	--	--	1
Australimusa					
M. maclayi	--	M	--	--	2
M. troglodytarum					
'Fehi'	F/M/S/VVS	M	F	--	3
'Fe'i'	F/M/S/VVS	M	F	--	4
Rhodochlamys					
M. ornata	M	S	--	--	5
M. velutina	--	S	--	--	6
Eumusa					
AA Group					
M. acuminata					
'Sucrier'	M/S	M	S	--	7
'Lady's finger'	F/M/S	M	S/F	--	34
'Lady's finger	F/M/S	S	S	--	35
'Lacatan'	F/M/S	M	S/F	--	36
'Lacatan'	F/M	M/VVS	S/M	M	8
'Lacatan'	F/M/S	M	S/F	--	9
subspecies microcarpa					
'Zebrina'	M/S	M	--	--	37
'Zebrina'	M/S	M	S	M	38
AAA Group					
'Gros Michel'					
'Amritsagar'	F/M/S	M	F	--	10
'Bluefields'	F/M/S	M	S/M	--	11

Table 2. (continued)

Section Species 1/ Clone	Px_1	Px_2	Px_3	Px_4	Zymogram 2/ Number
Eumusa (continued)					
'Red' 'Green Red'					
'Cuban Red'	F/M/S	M	S/M	--	41
'Cuban Red'	F/M	M	S	--	42
'Green Red'	F/M/S	M	S/M	--	12
'Iholena'					
'Iholena'	M/S	M	F	--	13
'Iholena'	M	M	--	--	43
'Haa Haa'	F/M/S	M	M	--	14
Cavendish Subgroup					
'Chinese'	F/M/S	M	S/M	--	44
'Chinese'	F/M/S	S	S/M	--	45
'Dichotomous Chinese	F/M/S	S	S	--	15
'Dichotomous Samoan'	F/M/S	S	S/M	--	16
'Williams Hybrid'	F/M/S	M	S/M	--	17
'Pisang Masak Hijau'					
'Hamakua'	F/M/S	M	S/M	--	47
'Hamakua'	F/M	M	S/M	--	48
'Lacatan' W.I.	F/M/S	M	S/M	--	18
'Monte Cristo'	--	M	S/F	--	49
'Monte Cristo'	M/S	M	S/F	--	50
'Robusta'					
'North'	F/M/S	M	S/M	--	51
'North'	M	M	S/M	--	52
'Valery'	F/M/S	M	S/M	--	19
AAAA Group					
'Golden Beauty'	F/M/S	M	S/F	--	20
AAB Group					
M. acuminata x M. balbisiana hybrids					
'Apple'	F/M	F/M	S/M	--	21

Table 2 (continued)

Section Species 1/ Clone	Px_1	Px_2	Px_3	Px_4	Zymogram 2/ Number
Eumusa (continued)					
'Brazilian'	--	M	--	--	22
'Rajapuri'	F/M/S	F/M	S/M	--	23
'Walha'	F/M/S	M	S/F	--	53
'Walha'	F/M/S	VF/M	S/F	M	54
Plantain Subgroup					
'Horn'	M/S	M	S/F	--	24
'Dwarf'	M/S	M	S/F	--	25
'Moongil'	M/S	M	S/M	--	26
Maoli					
'Haikea'	F/M/S	M	S/M	--	27
'Koae'	M/S	F	S/M	--	55
'Koae'	M/S	M	S	--	56
Popoulu					
'Hua Moa'	F/M/S	M	S/M	--	57
'Hua Moa'	M/S	M	S/M	--	58
'Lahilahi'	M/S	M	S	--	28
'Malei'	F/M/S	M	S	--	59
'Malei'	F/M/S	M	S/M	--	60
'Moa'	M/S	M	S/M	--	29
'Nou'	F/M/S	M	S/M	--	30
ABB Group					
'Ice Cream'	F/M/S	VF/M	--	--	31
'Monthan'	F/M/S	VF/M	F	--	32
'Pata Samoa'	M/S	VF/F	F	--	61
'Saba'	M/S	VF/M	S/M	--	33
Undetermined Group					
'Eslesno'	M/S	M	F	--	63
'Hapai'	F/M/S	M	S/F	M	64
'Tuu Gia'	F/M/S	M	F	M	65

Table 2. (continued)

Section Species 1/ Clone	Px_1	Px_2	Px_3	Px_4	Zymogram 2/ Number
Eumusa (continued) BB Group *M. balbisiana*	--	F	--	--	66

1/ Warner, 1970. List of Plants. Plant Science Instructional Arboretum. University of Hawaii.
2/ The zymograms shown graphically in Figures 2 to 9.

Systematic Polymorphisms Involving Peroxidases

The classification of the banana cultivars was taken from Warner (1970). A general description of the major sections of Musaceae is given in Appendix 1. The influence of the parental phenotype on Musa hybrids are listed in Appendix 2, and a description of the Hawaiian clones in Appendix 3.

Composite zymograms were then drawn from the data in Table 2. These values were the average of six gels (12 samples). The results are displayed in Figures 3 to 10.

M. coccinea, the single species tested from the Callimusa section of the genus, exhibited only a single, very slow moving band in the Px_2 zone. This very unique zymogram was distinct from all other species placing it in a category by itself.

Three clones of the Australimusa section were studied. M. maclayi was a unique Australimusa in that its bands were identical to the 'Brazilian' clone (AAB) in the Eumusa section and similar to M. coccinea, displaying only one band of activity. M. troglodytarum displayed activity in the Px_1, Px_2 and Px_3 regions but was distinct in that a new, very slow band was produced in Px_1. This was not seen in any other clones.

The Eumusa section presented the most complex peroxidase patterns. This section is divided into groups depending on ploidy and source of genomes. M. acuminata (AA), M. balbisiana (BB). The results generally agreed with the classifications that are found in Tables 1, 2 and 3 except for a few specific cases. The diploid types displayed a variety of bands in the various zones. A general

survey of the 'Sucrier' type diploids showed there was a general tendency for M-Px_2 and S-Px_3 bands to be present. Variations are displayed in Figures 3 and 6. The absence of a Px_3 band in one of the 'Zabrina' plants was noted. The true 'Lacatan' clone of the Philippines was the only assemblage of plants in this group that displayed Px_4 activity. These plants were definitely different from the 'Lacatan' clones.

Triploid (AAA) clones were very distinct in that the majority possessed a single M-Px_2 band, except in the case of dwarf clones. In the dwarf clones M-Px_2 becomes S-Px_2. The majority also showed all three bands in Px_1. In general, the subgroups within the triploid (AAA) group could not be distinguished from one another because of nearly identical zymograms.

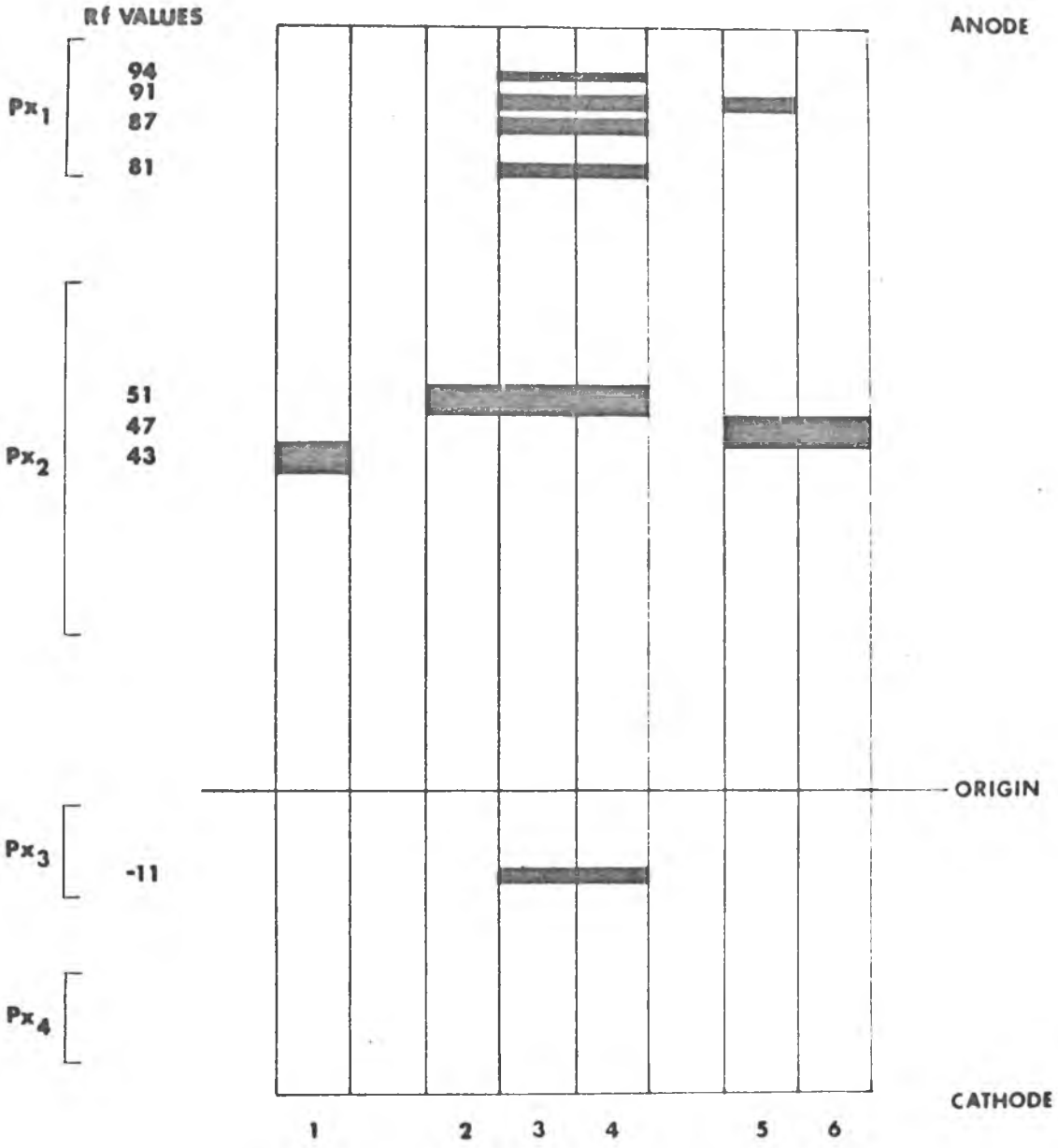
'Golden Beauty' of IC-2, a hybrid tetraploid of M. acuminata x 'Gros Michel' possessed uniform peroxidase bands which were identical to the 'Gros Michel' (AAA), except that the stain in Px_2 was slightly broader and more diffused than the triploid.

Triploids with AAB chromosome compliment displayed a wide variety of band variation. This group contained 0 to 3 bands in Px_1, 1 to 2 bands in Px_2, 0 to 3 bands in Px_3, and in one case the Px_4 band was present. The hybrid clones, 'Apple' (silk), 'Rajapuri' and 'Walha' exhibited two bands in Px_2, except for one plant of 'Walha'. The 'Brazilian' (pome) clone was unique in that there was no activity noted in Px_1 and Px_3 regions. The only peroxidase activity displayed was a single band in Px_2. This was an exception for this group with the zymogram almost a duplication

of the one of *M. maclayi*, in the Australimusa section. The Plantain subgroup was the most isozymically stable of this genotype. The 'Horn' and 'Popoulu' types were consistent in the Px_2 regions. The 'Maoli' type showed the double banding in Px_2.

The ABB genotypes were the most interesting as they displayed two bands (VF/M) in the Px_2 region. 'Pata Samoa' was an exception but was still easily identifiable from the rest. This double banding indicates that the A and B genomes do exhibit an effect on the peroxidase.

Figure 2. Zymograms of Specific Clones of Musa. (1) M. coccinea. (2) M. maclayi. (3) M. troglodytarum cv. 'fehi'. (4) M. troglodytarum cv. 'fe'i'. (5) ornata. (6) M. velutina.


Rf VALUES
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91
87
81
Px2
51
47
43
ORIGIN
Px3
-11
Px4
CATHODE
1
2
3
4
5
6

Figure 3. Zymograms of Specific Clones of Musa. (7) M. acuminata. (8) 'Lacatan'. (9) 'Lacatan'. (10) 'Amritsagar'. (11) 'Bluefield'. (12) 'Green Red'. (13) 'Iholena'. (14) 'Haa Haa'. (15) 'Dichotomous Chinese'.

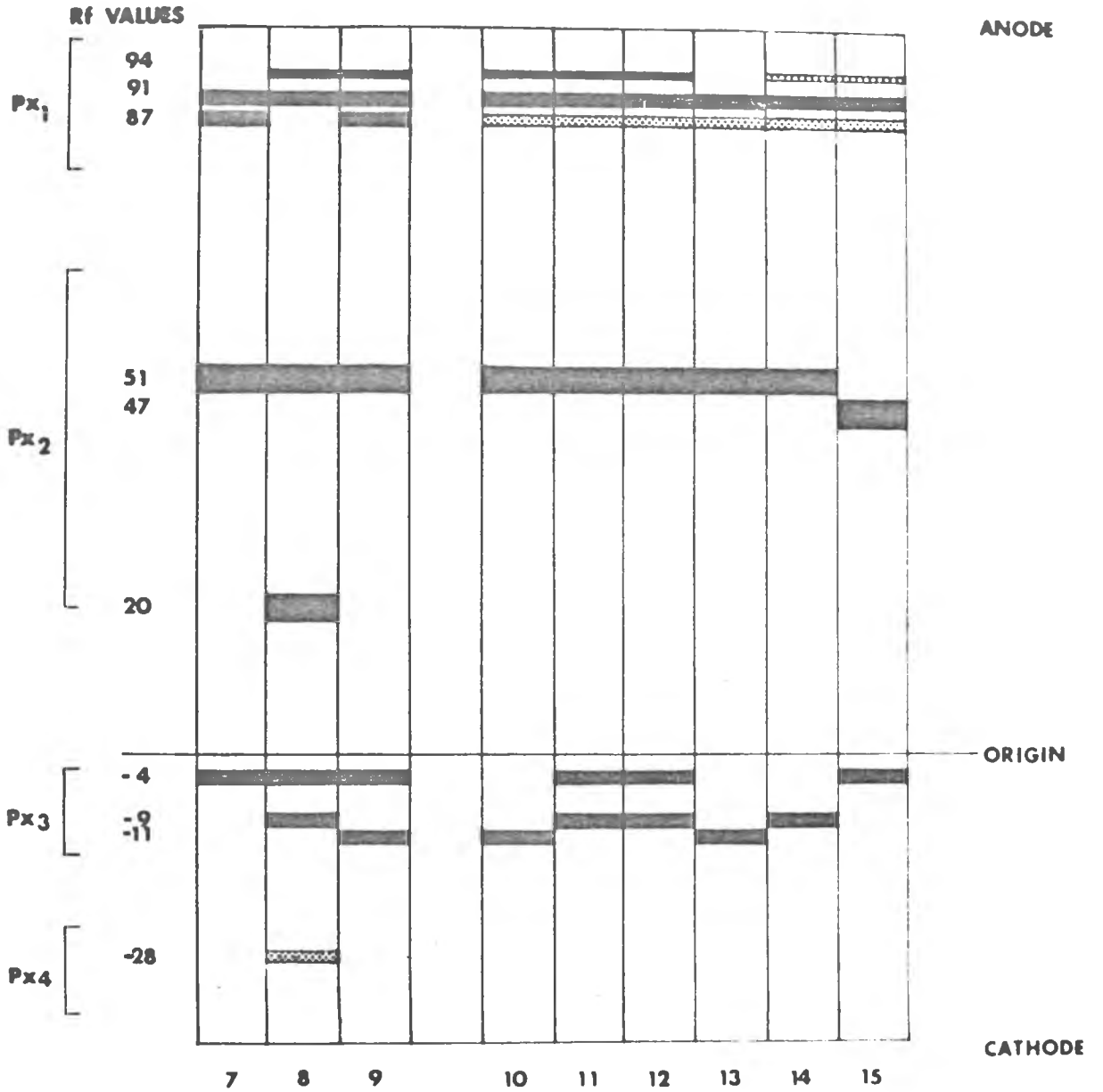
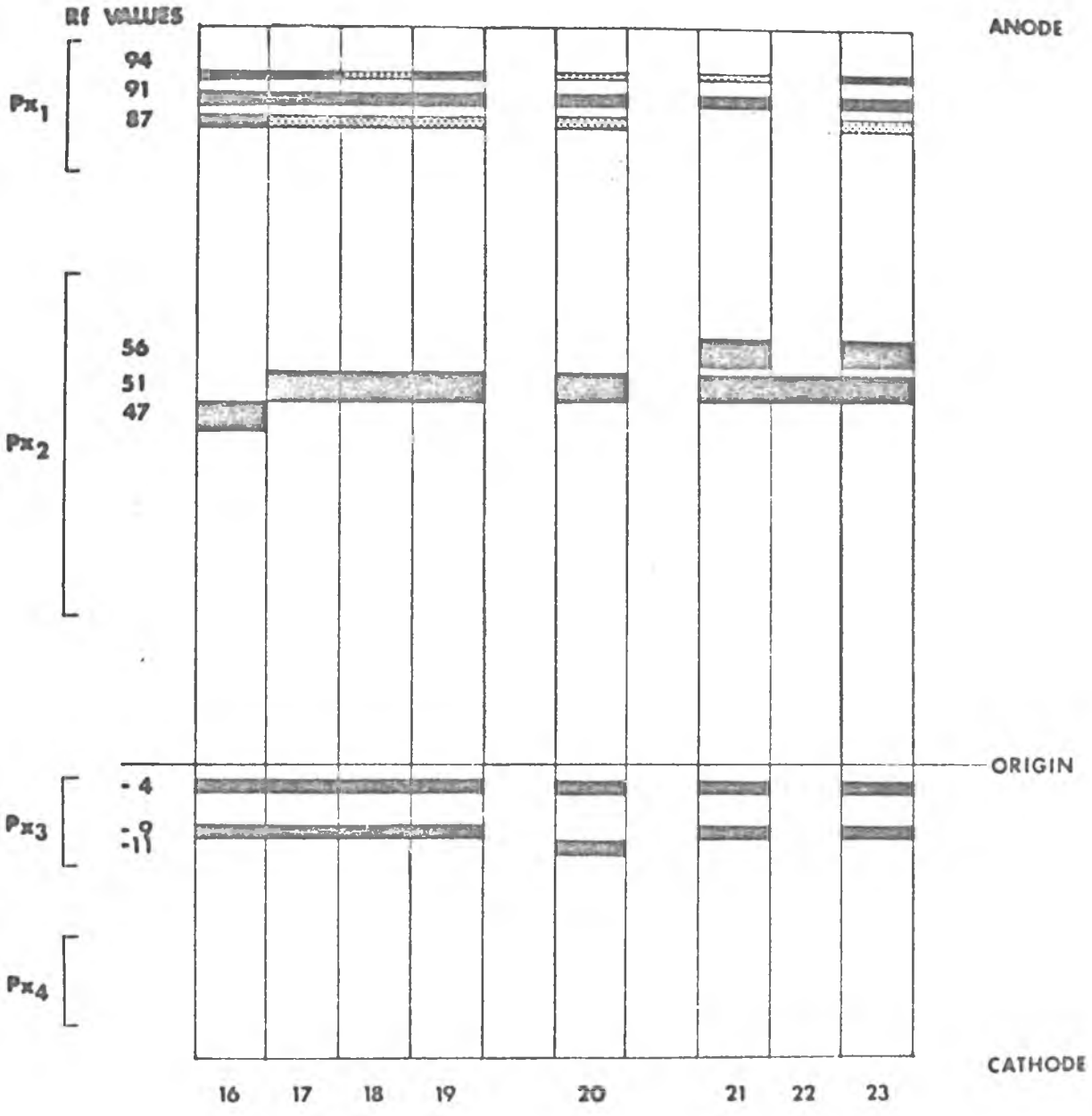

Rf VALUES
ANODE
94
91
87
Px1
51
47
Px2
20
ORIGIN
-4
-9
-11
Px3
-28
Px4
CATHODE
7
8
9
10
11
12
13
14
15

Figure 4. Zymograms of Specific Clones of Musa. (16) 'Samoan Dichotomous'. (17) 'Williams Hybrid'. (18) 'Lacatan' (West Indian). (19) 'Valery'. (20) 'Golden Beauty'. (21) 'Apple'.(Silk). (22) 'Brazilian' (Pome). (23) 'Rajapuri'.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47
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ORIGIN
- 4
Px3
-9
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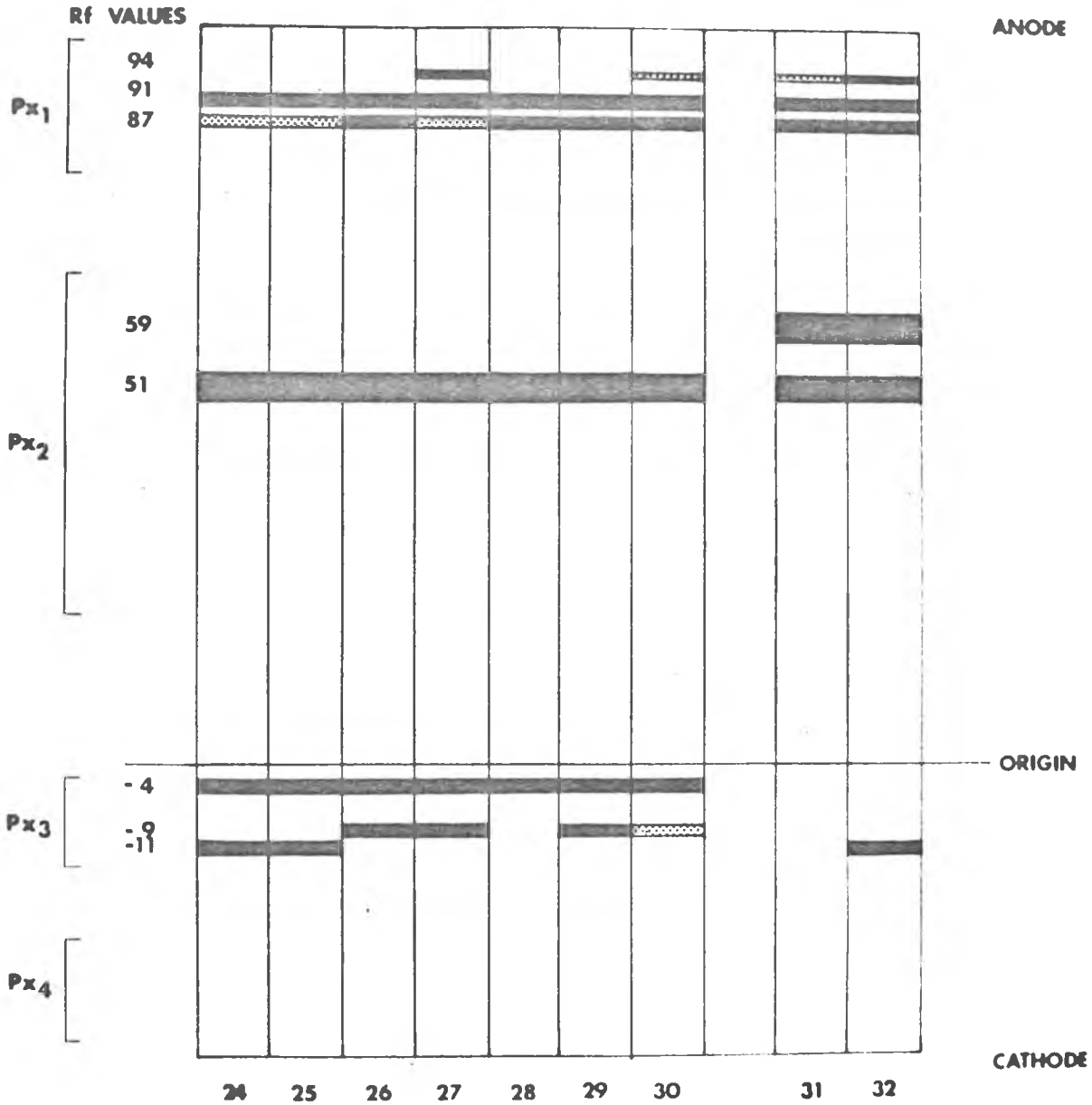
Px4
CATHODE
16
17
18
19
20
21
22
23

Figure 5. Zymograms of Specific Clones of Musa. (24) 'Horn' Plantain. (25) 'Dwarf' Plantain. (26) 'Moongil'. (27) 'Haikea'. (28) 'Lahilahi'. (29) 'Moa'. (30) 'Nou'. (31) 'Ice cream'. (32) 'Monthan'.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Px2
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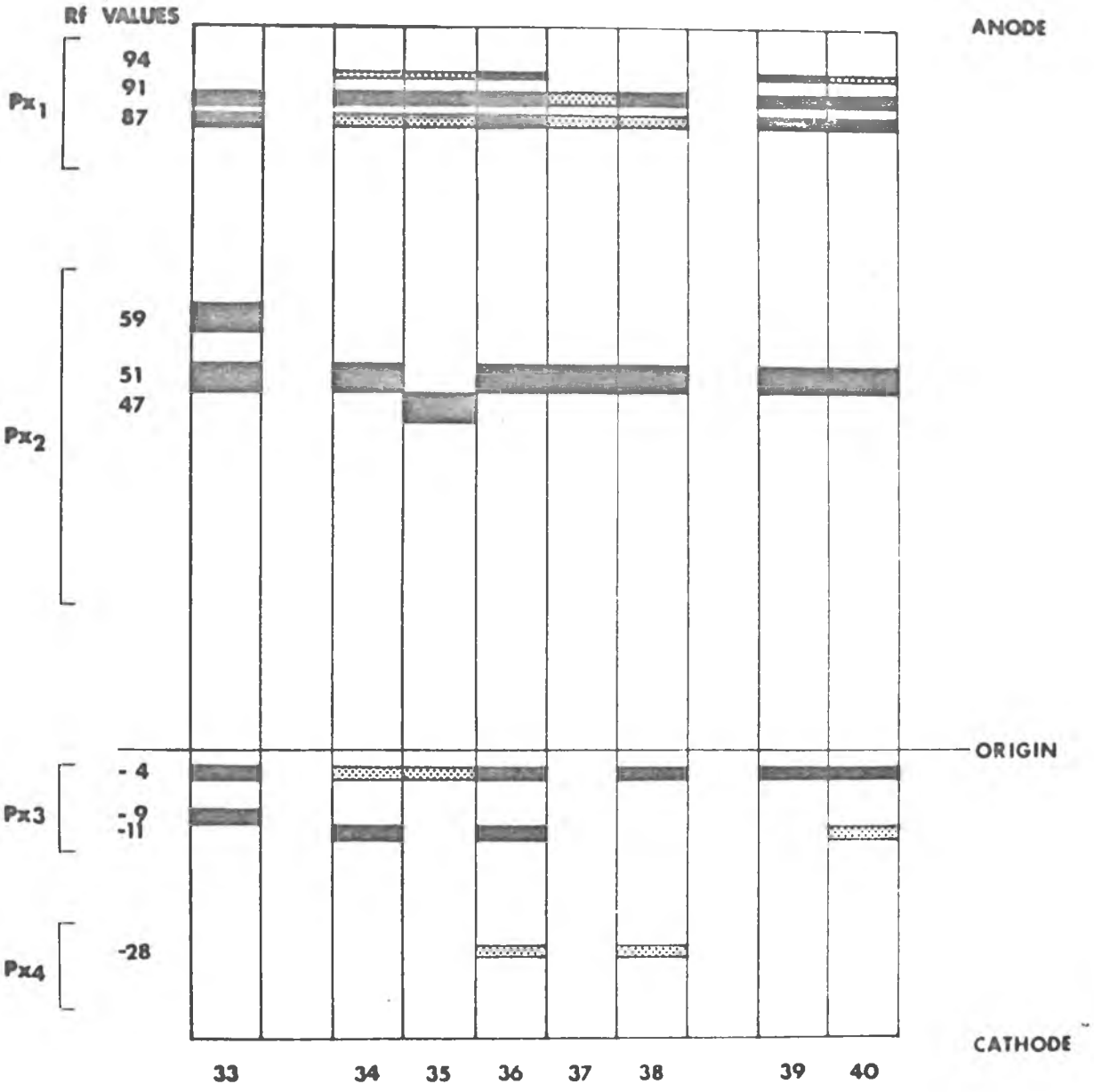
- 4
-9
-11
Px3
Px4
CATHODE
24
25
26
27
28
29
30
31
32

Figure 6. Zymograms of Specific Clones of Musa. (33) 'Saba'. (34) 'Lady's Finger'. (35) 'Lady's Finger'. (36) 'Lacatan'. (37) 'Zebrina'. (38) 'Zebrina'. (39) 'Cocos'. (40) 'Cocos'.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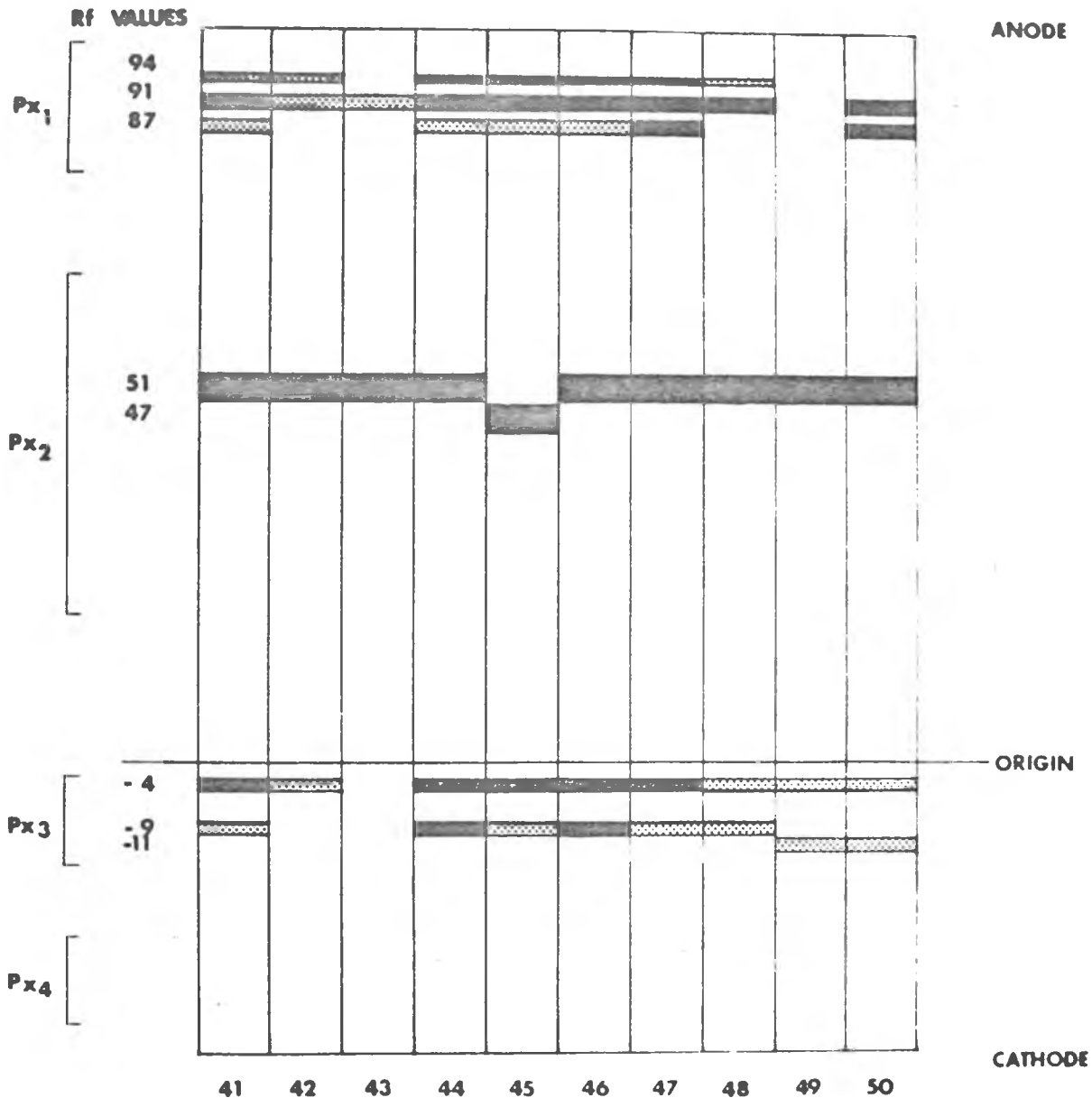
Px2
ORIGIN
- 4
-9
-11
Px3
-28
Px4
CATHODE
33
34
35
36
37
38
39
40

Figure 7. Zymograms of Specific Clones of Musa. (41) 'Cuban Red'. (42) 'Cuban Red'. (43) 'Iholena'. (44) 'Chinese'. (45) 'Chinese'. (46) 'Williams Hybrid'. (47) 'Hamakua'. (48) 'Hamakua'. (49) 'Monte cristo'. (50) 'Monte cristo'.


Rf VALUES
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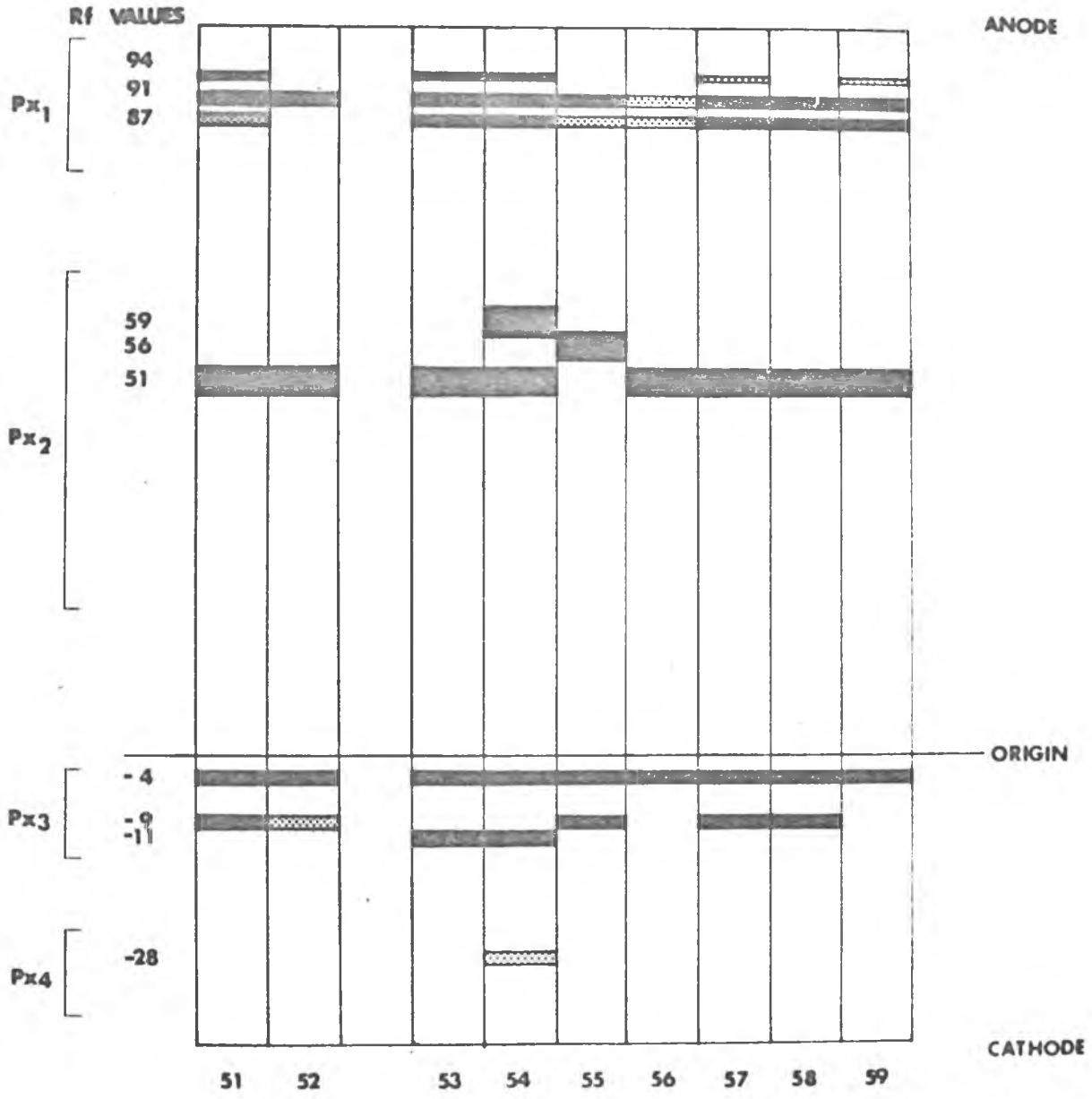
91
87
Px1
51
47
Px2
ORIGIN
- 4
-9
-11
Px3
Px4
CATHODE
41
42
43
44
45
46
47
48
49
50

Figure 8. Zymograms of Specific Clones of Musa. (51) 'North'. (52) 'North'. (53) 'Walha'. (54) 'Walha'. (55) 'Koae'. (56) 'Koae'. (57) 'Hua Moa'. (58) 'Hua Moa'. (59) 'Malei'.


Rf VALUES
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Px1
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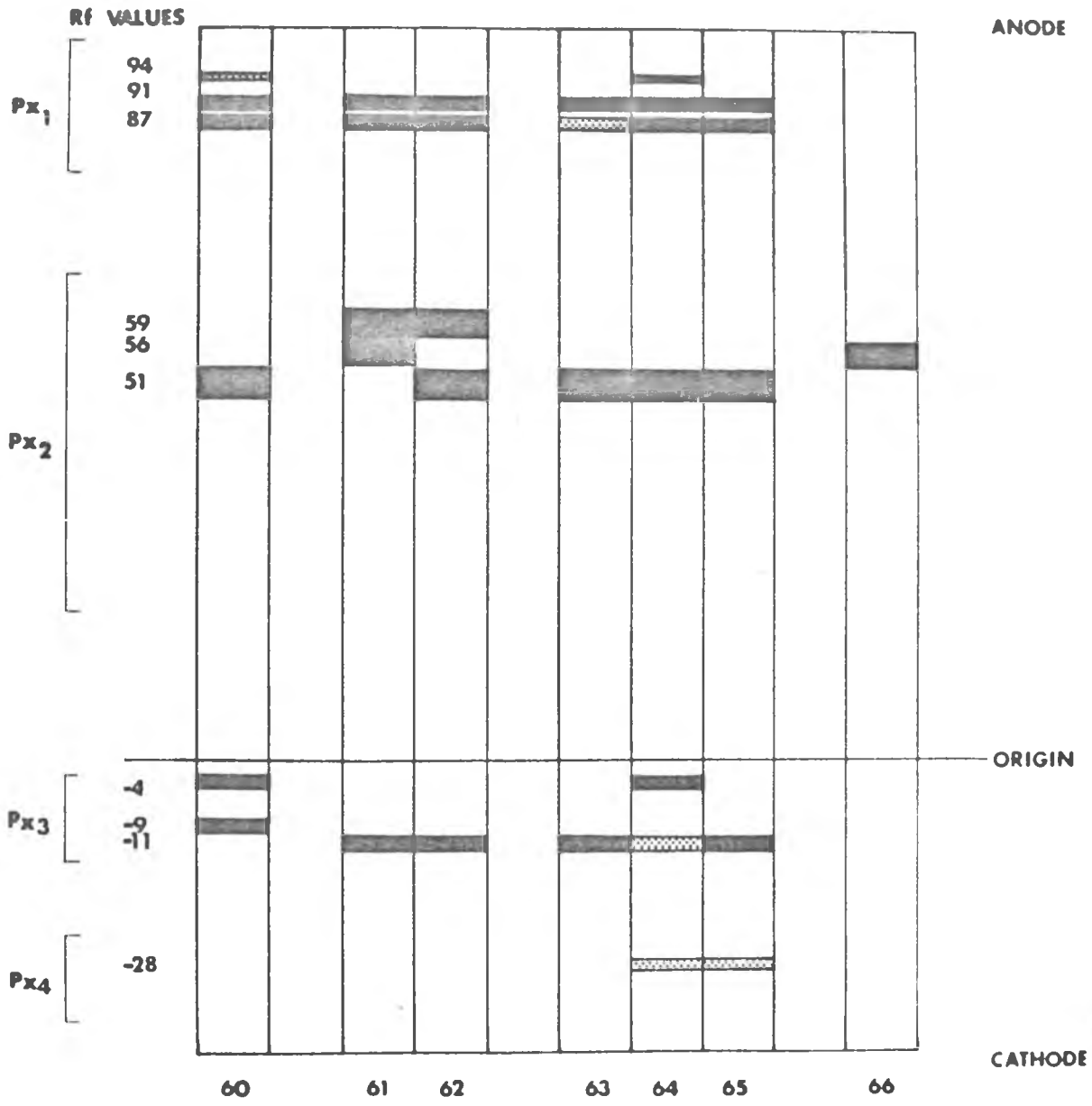
ORIGIN
- 4
- 9
-11
Px3
-28
Px4
CATHODE
51
52
53
54
55
56
57
58
59

Figure 9. Zymograms of Specific Clones of Musa. (60) 'Malei'. (61) 'Pata Samoa'. (62) 'Pata Samoa'. (63) 'Eslesno'. (64) 'Hapai'. (65) 'Tuu Gia'. (66) M. balbisiana.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-28
Px4
CATHODE
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66

CONCLUSIONS

These analyses suggest that an isozymic approach to systematic investigations in Musa is a useful and practical tool.

The Px_2 zone seemed to be the most useful in determining the genotypes of specific clones. M. acuminata with a medium ($M\text{-}Px_2$) band and M. balbisiana with a fast ($F\text{-}Px_2$) band were found in the Px_2 zones of their hybrids. The AAB genotypes displayed this to some extent, exhibiting fast and medium ($F/M\text{-}Px_2$) bands in specific cases. The most striking examples were the very fast and medium ($VF/M\text{-}Px_2$) bands consistently found in the ABB genotypes. The mixing of the slower A type and faster B type peroxidases in Px_2 raise some interesting questions. Why is the B genotype not easily detectable in an AAB triploid but easily detected in ABB types? Is the A genome dominant to the B genome?

The interpretations of the four peroxidase active zones were extremely difficult. There seemed to be a high degree of individual plant specificity in these zones. There was a variation in Px_3, from medium ($M\text{-}Px_3$) to fast ($F\text{-}Px_3$), between young and old tissues. Px_4 was consistently found in the root (Figure 1) and while its occasional presence in the leaf was unexplainable.

These peroxidase differences may be the result of genetic factors, causing slight alterations in the molecular structure of the peroxidase between clonal material or a natural variation among biological systems. A natural biological or an unnoticed

environmental factor would be capable of stimulating these differences. Any factor that would stimulate a metabolic change in the plant, such as disease which might cause the production of a new peroxidase band, which went unnoticed, would completely destroy the basis for a systematic investigation. Careful consideration was given to this fact. It was felt that working with plants from the same relatively small area of an Experimental Station where damage from pests and diseases are carefully controlled, was a safe approach for investigation. In this situation the plants are grown under practically identical conditions, hopefully reducing the chance for false zymograms. Samples were taken from areas outside the station and compared with identical materials from the collection area to determine if any discrepancies existed. None were found.

Small variations among identical plants were expected as a result of the methodology and natural variations within a population. The plants are listed separately in Figures 6 to 9. On inspection, it was determined that the majority of these differences were minor and statistically not significant. In some cases there was great variation. These large variations are difficult to explain. They may be due to improperly identified plant material or somatic mutation occurring in the sword suchers.

Some materials were so variable that composite zymograms could not be drawn. 'Father Leonore' and a 'Dichotymous Chinese' clone were in this category. In supposedly identical

plants, one displayed peroxidase activity in M-Px_2 and the other displayed all possible bands in Px_1, Px_2 and Px_3.

The plants of undetermined genotypes were examined and an attempt was made to match their zymograms with a zymogram of a known genotype. On examination of the 'Eslesno' clone, the zymogram displayed a similarity to known AAB types. Investigation into the literature revealed that 'Eslesno' was considered either AAB or ABB types (Simmonds 1970). Since all the known ABB displayed two bands in Px_2, the possibility of ABB was ruled out. It is suggested that 'Eslesno' is of the AAB genotype. The Hawaiian 'Hapai' clone, another undetermined genotype, displayed Px_4 activity. Because of the similarity of this clone and the known diploid clones, it is suggested that the 'Hapai' clone is diploid. The clone 'Tuu Gia', also undetermined as to genotype, displayed peroxidase activity similar to 'Hapai'. For similar reasons it is suggested that 'Tuu Gia' is diploid.

This is a brief survey of the very complex systematics of the Musa species. A very practical followup to this investigation would be additional zymograms from various other tissues of the plants and investigations of other isozymes such as esterases, catalases and phosphatases.

SUMMARY

1. Peroxidase isozyme technique on Musa spp. seems to be a valuable tool for systematic studies. Of the four major peroxidase active zones Px_2 was the most consistent and useful zone in these investigations, with noted exceptions. In all, a total of 15 peroxidase bands were isolated.

2. M. balbisiana was easily identified with the only activity noted in the fast (F-Px_2 band. This was a good parental type genetic marker, as the majority of the edible bananas are thought to be hybrids of M. acuminata x M. balbisiana. M. acuminata displayed more peroxidase activity, with bands in the Px_1 and Px_3 regions, along with a medium (M-Px_2) band.

3. The ABB genotypes in the Eumusa section were most readily identifiable with a double banding in the Px_2 region very fast and medium (VF/M. This indicated influences from both parental types, M. acuminata (M) and M. balbisiana (F). The other genotypes were more difficult to interpret.

4. By matching zymograms of three unknown clones with general characteristics of known genotypes, the following results are suggested: 'Eslesno' (AAB), 'Hapai' (diploid AA), 'Tuu Gia' (diploid AA).

APPENDIX

APPENDIX 1.. Sections of the Genus Musa. [1]

A. Chromosome number x=11. Bracts usually more or less sulcate, often more or less glaucous, rarely or never polished, convolute or more or less imbricated in the bud, usually strongly revolute on fading. Seeds occasionally subglobose, more often dorsiventrally compressed, sometimes lenticular, smooth, tuberculate or irregularly angulate with a marked or obsolete umbo opposite the hilum.

1. Inflorescence pendent or semi-pendent from the first, the fruits reflexing in development towards the base of the rachis. Flowers many to a bract, in two series. Bracts commonly dull-colored, green brownish or dull purple. Pseudostems commonly exceeding three meters high.

Section EUMUSA

2. Inflorescence erect, or at least at the base, so that the fruits do not reflex in development but point towards the apex of the rachis. Flowers few to a bract, usually in a single series. Bracts brightly colored, often red. Pseudostems commonly less than three meters high.

Section RHODOCHLAMYS

B. Chromosome number x=10. Bracts plane, firm in texture, polished on the outside, rarely or never glaucous, strongly imbricate in the bud, not or slightly revolute on fading.

3. Seeds subglobose or more or less dorsiventrally compressed, smooth, striate, tuberculate, or irregularly angulate, with a marked or obsolete umbo opposite to the helium corresponding to a small perisperm chamber within.

Section AUSTRALIMUSA

4. Seeds cylindrical, barrel-shaped, or top-shaped, marked externally by a transverse line or groove, above which they are warted, tuberculate or variously patterned, below usually smooth; internally with a well-developed perisperm chamber above the same line, this chamber empty in the ripe seed.

Section CALLIMUSA

1. Simmonds 1962.

APPENDIX 2.

Characters Used in Taxonomic Scoring of Banana Cultivars [2/]

Character	*M. acuminata*	*M. balbisiana*
Pseudostem colour	More or less heavily marked with brown or black blotches	Blotches slight or absent
Petiolar canal	Margin erect or spreading, with scarious wings below, not clasping pseudostem	Margin inclosed, not winged below, clasping pseudostem
Peduncle	Usually downy or hairy	Glabrous
Pedicels	Short	Long
Ovules	Two regular rows in each loculus	Four irregular rows in each loculus
Bract shoulder	Usually high (ratio <0.28)	Usually low (ratio >0.30)
Bract curling	Bracts reflex and roll back after opening	Bracts lift but do not roll
Bract shape	Lanceolate or narrowly ovate, tapering sharply from shoulder	Broadly ovate, not tapering sharply
Bract apex	Acute	Obtuse
Bract colour	Red, dull purple or yellow outside; pink, dull purple or yellow inside	Distinctive brownish-purple outside; bright crinson inside
Colour fading	Inside bract colour fades to yellow towards the base	Inside bract colour continuous to base

APPENDIX 2. (continued)

Character	M. acuminata	M. balbisiana
Bract scars	Prominent	Scarcely prominent
Free tepal of male flower	Variably corrugated below tip	Rarely corrugated
Male flower colour	Creamy white	Variable flushed with pink
Stigma colour	Orange or rich yellow	Cream, pale yellow or pale pink

2/

APPENDIX 3. Hawaiian Varieties of *Musa*.[2]

Maoli Group: Varieties of the Maoli group have unusually large plants requiring great quantities of moisture and protection from the wind. The bunches of fruit are large and compact; individual fruits are long, well-filled to the ends, and covered with thick, heavy skin, which, on ripening, turns to a rich, waxy yellow color. The pedicel is long and the flesh is yellow. Floral parts are slightly tinged with pink. The varieties are: Maiamaoli, Hai, Haikea, Maniula, Kaualau, Eleele, Koae, Mahoe, Puhi, Eka, and Iho-u.

Iholena Group: Varieties of the Iholena group are identified by their erect trunks, long arched fruiting stems, and small or medium sized bunches, rarely weighing over 50 pounds. Individual fruits stand out at nearly right angles to the stem, and usually are angular, tapering toward the apex; the skin is thick and yellow at maturity, the flesh is of light pink salmon color, and the rachis is rather long. The flowers are white or cream colored. Varieties included are: Iholena, Kapua, and Lele.

Popoulu Group: Varieties of the Popoulu group are characterized by their medium height and compact bunches of fruit, the fingers of which are short and thick, without angles, and set at right angles to the axis of the bunch. At full maturity, the skin is very thin and the pulp is a light salmon color. Included in this group are: popoulu, Kaio, and Moa.

2. Pope 1926.

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